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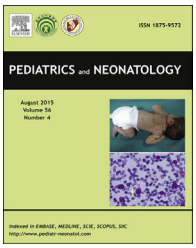

ORIGINAL ARTICLE

Importance of Direct Antiglobulin Test (DAT) in Cord Blood: Causes of DAT (+) in a Cohort Study

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Key Words
ABO incompatibility;
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hyperbilirubinemia;
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phototherapy

Background: The direct antiglobulin test (DAT) is the cornerstone of the diagnosis of hemolytic disease of the newborn (HDN). The aim of this study was to review the incidence and causes of positive DAT in cord blood in relation to development of HDN.

Methods: We retrospectively reviewed all results of DAT, which is routinely performed in cord blood samples, along with the laboratory and infants' medical records.

Results: DAT was positive in 70/2695 (2.59%) cases. In 64/70 (91.43%) cases, DAT positivity was attributed to ABO incompatibility. There were 50/218 (22.93%) DAT (+) cases in the A/O group and 13/97 (13.40%) cases in the B/O group ($p = 0.0664$). Two DAT (+) cases were attributed to maternal alloimmunization (anti-Fya and anti-JKb, respectively), and one to maternal IgG autoantibodies that developed after methyldopa treatment. Among the 70 DAT (+) cases, 30 (42.86%) cases required phototherapy with no difference between the A/O and B/O groups. The duration of phototherapy in the B/O group was significantly longer than in the A/O group ($p = 0.024$). There was a trend of correlation of increasing strength of DAT positivity with phototherapy need. No false positive DAT case was detected.

Conclusions: Although ABO incompatibility remains the main reason of DAT (+), other causes (e.g., alloimmunization, drugs) should also be explored. The relevant impact of DAT (+) on HDN development should be considered.

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1. Introduction

The direct antiglobulin test (DAT) is a screening test for antibodies present in an individual's red cells and is used to diagnose autoimmune hemolytic anemias as well as the hemolytic disease of the newborn (HDN).[1] A positive DAT in a newborn results from the transplacental transfer of IgG antibodies, which are present in maternal serum and directed against antigens on fetal and neonatal red blood cells (RBCs). Such antibodies may cause destruction of neonates' RBCs and shorten their life span, leading to clinical manifestations of HDN and various degrees of hyperbilirubinemia and anemia.[1]

The factors that can lead to a positive DAT in neonates are mainly the ABO incompatibility between the newborn and the mother, maternal alloimmunization, and very seldom maternal autoimmune hemolytic anemia.[1] ABO incompatibility with a positive DAT is considered a major risk factor for the development of severe hyperbilirubinemia and neurotoxicity.[2,3] By contrast, some studies report that the positive DAT has only a poor predictive value for severe hyperbilirubinemia.[4–8]

This study aimed to explore in a cohort of newborns the incidence of DAT positivity, evaluate its strength, identify its causes, and examine its association with hyperbilirubinemia, need for phototherapy, and transfusion for the newborns.

2. Methods

Following the guidelines of the Ethics Committee, at admittance to the Aretaieion University Hospital, all parturients sign an informed consent for indispensable laboratory tests to be performed, including ABO/Rhesus D group and DAT in cord blood. In this respect, we retrospectively reviewed all results of DAT, which was routinely performed in cord blood samples of all infants born between January 2011 and December 2012.

During this period, a total of 2695 cord blood samples were analyzed. From the Blood Bank records, the following data were evaluated: DAT results, strength of DAT positivity, infant/maternal ABO and Rhesus D group, antibody screening, and antibody identification in cases of maternal alloimmunization. A retrospective review of the charts of infants with a positive cord blood DAT was also performed. The following characteristics were recorded: sex, gestational age, mode of delivery, relevant antenatal and delivery data, (maternal medical history) treatment with phototherapy (duration), peak serum total bilirubin (STB), and transfusion history. The bilirubin level was plotted to the hour-specific bilirubin nomogram based on Bhutani et al[9] as referred by Schutzman et al.[10] The risk zone on the nomogram was characterized as follows: zone a, low risk: $<40^{th}$ percentile; zone b, low-intermediate risk: $40–75^{th}$ percentile; zone c, high-intermediate risk: $76–95^{th}$ percentile; and zone d, high risk: $>95^{th}$ percentile. Phototherapy was implemented according to the guidelines of the neonatal department, based on the 2004 American Academy of Pediatrics along with the revised 2011 American Academy of Pediatrics criteria, for the management of hyperbilirubinemia in newborns.[2,11]

Cord blood was collected by puncturing the umbilical vein with needle and a syringe to avoid Wharton's jelly contamination. DAT was carried out using an automated method (BIOVUE INNOVA Ortho Clinical Diagnostics, High Wycombe, UK) according to the manufacturer's instructions and was characterized as negative, weak (equal to 0.5+), 1+, 2+, 3+, and 4+. Blood group typing was performed using standard blood bank techniques (slide or tube test).

Statistical analysis was performed using the *t* test for continuous data, and Fisher's exact test or the chi-square test for categorical data as appropriate. Data were analyzed with the SPSS.16 software. A p value of 0.05 or less was considered statistically significant.

3. Results

Among the 2695 neonates born between January 2011 and December 2012, 1074 (39.85%) neonates were of group A, 407 (15.10%) of group B, 1105 (41.00%) of group O, and 109 (4.04%) of group AB. ABO incompatibility between neonates and mothers was found in 481 (17.85%) cases. Of these, 218 neonates of group A and 97 of group B were born to group O mothers (A/O, 218; B/O, 97). The remaining ABO incompatible cases identified were B/A, 38 cases; AB/A, 48 cases; A/B, 43 cases; and AB/B, 37 cases. The ABO neonatal/maternal compatibility status could not be determined in 296 cases (208 neonatal group A, 11 neonatal group AB, and 77 neonatal group B), as the study was retrospective and data matching mothers and infants of different surnames was lacking. All the remaining 1918 (71.17%) cases were considered ABO compatible (A/A, 537; A/AB, 68; AB/AB, 13; B/AB, 38; B/B, 157; O/A, 223; O/B, 111; O/O, 539; O/unknown maternal ABO group, as stated above, 232).

DAT was found to be positive in 70/2695 (2.59%) cases. In 64/70 (91.43%) cases, DAT positivity was attributed to ABO incompatibility. There were 50/218 (22.93%) DAT positive cases in the A/O group, 13/97 (13.40%) in the B/O group, and 1/38 (2.63%) in the B/A group. The incidence of DAT positivity was higher (although it did not reach statistical significance) in the A/O group as compared to the B/O group ($p = 0.0664$).

In two cases, DAT positivity was the result of maternal alloimmunization. The first case of alloimmunization was a mother para 1 (B RhD positive), compound heterozygote for sickle cell anemia and beta thalassemia. She had a history of alloimmunization with anti-Fya and anti-JKb owing to past transfusions, of which only anti-Fya was still detectable. The titer of anti-Fya never exceeded 64 during pregnancy. The neonate (B RhD positive), delivered by cesarean section at 33 weeks of gestation, was Fya antigen positive and had a DAT positive (1+) result. The presence of anti-Fya, as the cause of DAT positivity, was confirmed via an elution test. The neonate did not suffer hemolysis and was discharged on Day 7 in good health.

The second case of alloimmunization was a mother para 3 (A RhD positive) without history of past transfusions, who uneventfully delivered a male infant. The neonate (O RhD positive) had a strong positive DAT (4+). An antenatal antibody screening test of the mother was not available and the one performed at labor was positive. The alloantibody identified had anti-Jkb specificity. The neonate was Jkb

antigen positive, and the eluate obtained from neonatal red cells confirmed the diagnosis of HDN due to anti-Jkb. It was the only 4+ DAT case in our cohort. The neonate developed hemolysis and jaundice and responded well to phototherapy.

One additional DAT positive case was an A RhD negative male infant born to an AB RhD positive mother with a history of methyldopa treatment, owing to hypertension. Both the mother and the neonate had a DAT positive result (3+), and the eluate obtained in both cases revealed a nonspecific reaction. The neonate developed hemolysis and jaundice and responded well to phototherapy.

As for the remaining three DAT positive cases, in two cases concerning neonates of group A, the ABO status of the mother was not available, whereas in the other A/A case DAT positivity could not be attributed to any kind of alloimmunization because of missing data of the mother. No case of HDN due to maternal anti-D alloimmunization was identified in our study.

Among the 70 DAT positive cases, the strength of DAT positivity was distributed as follows: 0.5+, 9/70 (12.86%); 1+, 33/70 (47.14%); 2+, 23/70 (32.86%); 3+, 4/70 (5.71%); 4+, 1/70 (1.43%). The strength of DAT positivity in the A/O and the B/O groups was similar. No case with DAT 4+ positivity was detected in the ABO incompatibility group.

Among the 70 DAT positive cases, applying all to neonates with gestational age >36 weeks, 30 (42.86%) met the criteria and received phototherapy treatment. Twenty-two of 50 (44%) infants belonged to the A/O group and six of 13 (46.2%) belonged to the B/O group, with the difference not being significant ($p = 1$). However, the duration of phototherapy in the B/O group (mean ± SD, 34.42 ± 45.49 hours) was significantly longer than in the A/O group (mean ± SD, 14.56 ± 19.34 hours; $p = 0.024$). The two additional cases that received phototherapy were the newborns with HDN due to anti-JKb and to maternal autoantibodies as a result of methyldopa treatment of the mother. None of these cases required an exchange transfusion or intravenous immunoglobulin.

Fifteen out of seventy (15/70, 21.43%) infants with a positive DAT developed clinically significant jaundice with an STB value > 95th percentile for hour of life (high-risk, zone d). The two subgroups, A/O (11/50) and B/O (4/13), did not differ in terms of development of hyperbilirubinemia ($p = 0.7191$).

The strength of DAT positivity in relation to the risk zone (according to STB percentile for hour of life) and cases that met the phototherapy criteria are depicted in Table 1.

There was a trend of correlation of increasing DAT positivity strength with increasing need for phototherapy as shown in Figure 1. Further analysis revealed a linear relation between the percentage of phototherapy-treated neonates (PPTN) and the DAT positivity strength: PPTN = 0.19 + DAT × 0.20 ($p < 0.05$ both for intercept and slope, and $R^2 = 98.31\%$). There was also a trend of correlation of increasing DAT positivity strength with increased duration of phototherapy (mean values). The duration of phototherapy (mean hours) was 11.89 hours in the 0.5+ DAT group, 15.09 hours in the 1+ DAT group, 21.31 hours in the 2+ DAT group, 22.00 hours in the 3+ DAT group, and 33.00 hours in the 4+ DAT group. For those data, there was a statistically significant relation between the duration of phototherapy (mean hours) per neonate (DPPN) and DAT positivity strength ($p < 0.05$ both for intercept and slope, and $R^2 = 93.04\%$), specifically DPPN = 9.20 + DAT × 5.46.

Table 1 Number of neonates in the low-risk zone (zone a), the low-intermediate risk zone (zone b), the high-intermediate risk zone (zone c), and the high risk zone (zone d) of the nomogram in relation to the strength of DAT positivity, and cases that met phototherapy criteria.

Risk zone distribution	DAT positivity					DAT positive cases (%)	Cases meeting phototherapy criteria (%)
	0.5+	1+	2+	3+	4+		
a	6	18	8	1	0	33 (47.14)	0 (0)
b	1	3	2	1	1	8 (11.43)	5 (62.5)
c	1	6	5	0	0	12 (17.14)	11 (91.66)
d	1	6	6	2	0	15 (21.43)	14 (93.33)
No data*	0	0	2	0	0	2* (2.86)	3*
Total	9	33	23	4	1	70 (100)	30 (42.86)

* Three infants were transferred at birth to Referral Centers as they presented severe congenital heart disease (2 cases: risk zone distribution and phototherapy unknown) and severe dermatological problems (1 case: risk zone distribution d, phototherapy unknown).

4. Discussion

In this study, we retrospectively analyzed all DAT results routinely performed in cord blood samples of all infants born at the Aretaieion Hospital and explored the reasons that led to DAT positivity, as well as its impact on neonatal outcome.

The ABO incompatibility between neonate and mother was 17.85%, in accordance with previous reports.[12] The higher frequency of A/O versus B/O incompatibility was also in accordance with the ABO group distribution in Greece.[13]

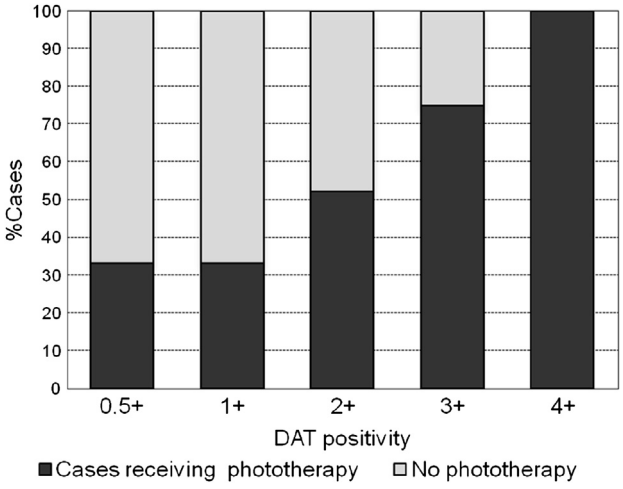

Figure 1 Direct antiglobulin test (DAT) positivity (DAT 0.5+, 1+, 2+, 3+, 4+) in relation to need for phototherapy.

The incidence of a positive DAT in newborns was 2.59%, a percentage similar to that (2.3%) reported by Dillon et al[14] but lower than that (3.5%) of Hershel et al.[15] Both studies applied the same methodology as the current study.[14,15] In accordance with previous studies, the current one presented ABO incompatibility in 91.43% of positive DAT cases,[15] and the incidence of DAT positivity was slightly higher in neonates of group A/O (22.93%) when compared to group B/O (13.40%; $p = 0.0664$).[6,16]

In our cohort, two cases of DAT positivity resulted from alloimmunization of the mother: one B/B case was due to anti-Fya and one O/A case was due to anti-JKb. The prevalence of anti-Fya alloantibody in pregnant women is very low (ranging from 0.01% to 5.4%), and although the neonate in our study did not suffer hemolysis, there is a potential to cause significant fetal and newborn hemolysis.[17] Most of the few case reports of anti-Jkb–related HDN described in the literature developed a mild clinical course, as in our case, but rare cases with severe HDN have also been reported.[18,19]

One additional A/AB case was attributable to maternal IgG autoantibodies that developed during pregnancy as a result of methyldopa treatment. Methyldopa can lead to a positive DAT in about 20% of treated patients, but autoimmune hemolytic anemia develops in only 2% of patients.[20] However, IgG autoantibodies against RBCs can cross the placenta and cause HDN. This case is, to our knowledge, the second in the literature.[21]

In our study, no case of positive DAT was attributed to anti-D passive or immune alloimmunization of the mother. In the A/O group with a positive DAT, there were four cases of Rhesus D positive infants born to Rhesus D negative mothers. All four mothers had a negative screening test result during pregnancy and at labor, and no history of prenatal Rhesus immune globulin administration. Thus, DAT positivity was attributed to ABO incompatibility, and this was also confirmed by elution tests. It has been shown that the introduction of routine antenatal Rhesus immune globulin prophylaxis led to an increase of false positive DAT results in cord blood samples that were poorly predictive of subsequent hyperbilirubinemia.[14,22]

However, there were three DAT positive cases in which DAT positivity could not be explained owing to a lack of data regarding the mother. We do not regard these cases as false positives but as true positive ones, where the cause of DAT positivity could not be clarified. In our study, no false positive DAT cases were detected.[22,23] All weak positive DAT cases (0.5+) were attributed to ABO incompatibility and had relevant clinical impact. Additionally, it is worth mentioning that DAT accuracy could be constrained owing to a degree of subjectivity in the observer's judgment. The use of fully automated microtube gel methods could overcome such problems along with technical pitfalls that could result in a false positive DAT.[24]

Fifteen of 70 (21.43%) neonates with a DAT-positive result developed hyperbilirubinemia (as defined by STB $>95^{th}$ percentile for hour of life) and 30/70 (42.86%) cases met the criteria and were treated with phototherapy. This is higher than the phototherapy incidence among DAT-positive neonates reported by Schutzman et al[10] (12.9%), but similar to that reported by Kaplan et al[25] (49.4%), and may be attributed to variant phototherapy criteria in neonatal units.

The incidence and the severity of hyperbilirubinemia between A/O and B/O subgroups differs among published studies, with many investigators suggesting that clinical severity of HDN does not differ in the two subgroups, and others proposing that B/O infants are at increased risk for HDN and in need of intense treatment. In our study, similar proportions of A/O and B/O infants met the criteria and received phototherapy or developed clinically significant jaundice (STB $>95^{th}$ percentile for hour of life). However, the duration of phototherapy (hours) received by neonates in the B/O group was higher than in the A/O group ($p = 0.024$). This finding could possibly suggest a slight difference in clinical severity of HDN in the B/O group, but cannot definitively document that B/O heterospecific neonates are at higher risk than their A/O counterparts.[16,25–28]

The role of DAT screening in predicting HDN has been debated over the past years.[6,8,29,30] The predictive value of the DAT positivity was not assessed in this retrospective study, because only the DAT positive cases were taken into consideration. However, it was found that among the DAT (+) cases, a trend of increasing DAT positivity strength with increasing need for phototherapy might exist as shown in Figure 1. In addition, a similar trend of increasing DAT positivity strength with increased duration of phototherapy may also exist. The main limitation of this result is related to the small number of cases encountered with 3+ and 4+ DAT.

In conclusion, although several reports have proposed that routine cord blood DAT testing is not necessary, according to our current retrospective study, its impact on the newborn cannot be overlooked. Nevertheless, DAT testing cannot replace STB measurements early in life. Furthermore, although ABO incompatibility accounts for the majority of DAT positive cases, other causes should be also considered. Maternal screening tests and a careful look at the history of drug administration during pregnancy could identify other important but rare causes of DAT positivity, aiding maternal and neonatal management. Finally, prospective studies including cost–benefit ones with defined end points would be helpful.

Conflicts of interest

The authors declare that they have no financial or nonfinancial conflicts of interest related to the subject matter or materials discussed in the manuscript.

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